For life science research only.
Not for use in diagnostic procedures.

Hygromycin B
from *Streptomyces hygroscopicus*

Version: 21
Content Version: December 2020

Solution

Cat. No. 10 843 555 001	1 g 20 ml

Store the product at +2 to +8°C.

1. General Information

1.1. Contents

Vial / Bottle	Label	Function / Description	Content
1	Hygromycin B, 50 mg/ml	In phosphate-buffered saline (PBS), filtered through a 0.2 µM pore-size membrane.	1 vial, 1 g, 20 ml

1.2. Storage and Stability

Storage Conditions (Product)

When stored at +2 to +8°C, the product is stable through the expiration date printed on the label.

Vial / Bottle	Label	Storage
1	Hygromycin B	Store at +2 to +8°C.

1.3. Additional Equipment and Reagent required

Standard Laboratory Equipment

- Microplates, tissue-culture grade
- Petri dishes, tissue-culture grade

Culture Medium

- The use of a chemically defined or serum-containing medium without addition of antibiotics, optimally meeting the specific media needs of the particular cell type or cell line to be cultured.

For Cell Viability

- Cell Proliferation Kit I (MTT)*
- Cell Proliferation Kit II (XTT)*
- Cell Proliferation Reagent WST-1*

1.4. Application

Hygromycin B is used for the selection of:
- A wide variety of pro- and eukaryotic cells, stably transfected with the hygromycin-resistance gene,
- as well as for the maintenance of the hygromycin phenotype of resistant cells.

2. How to Use this Product

2.1. Before you Begin

Safety Information

Laboratory procedures

- Handle all samples as if potentially infectious, using safe laboratory procedures. As the sensitivity and titer of potential pathogens in the sample material varies, the operator must optimize pathogen inactivation by the Lysis / Binding Buffer or take appropriate measures, according to local safety regulations.
- Do not eat, drink or smoke in the laboratory work area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats and eye protection, when handling samples and kit reagents.
- Wash hands thoroughly after handling samples and reagents.

Waste handling

- Discard unused reagents and waste in accordance with country, federal, state, and local regulations.
- Safety Data Sheets (SDS) are available online on dialog.roche.com, or upon request from the local Roche office.

2.2. Protocols

Determination of Hygromycin B Concentration

Cells can escape selection if the antibiotic is used at too low concentrations or if the plating density is too high. The sensitivity of non-resistant cells also depends on the proliferative activity of the cells. Cells rapidly proliferating are killed faster than those that are slowly proliferating. Ideally, control cells should die within one week after addition of the antibiotic, allowing colonies of resistant cells to form within 10 to 14 days.

The following protocol is a general guideline that may be modified according to the respective test system.
i *For selection of transfected Hygromycin B-resistant cells, use a concentration of Hygromycin B that completely blocks growth of sensitive, non-transfected cells.*

1. Plate non-transfected cells to be tested at a concentration of 50 to 200 cells/well in 200 µl culture medium containing various amounts of Hygromycin B, such as 50 to 1,000 µg/ml into microplates (tissue-culture grade, 96-wells).

2. Incubate cell cultures for 10 to 14 days.

3. After 5 to 7 days, replace culture medium with fresh culture medium containing the respective amounts of Hygromycin B, if required.
   i *A replacement of culture media containing Hygromycin B after 5 to 7 days is only necessary if nutritional compounds are supposed to be depleted by the cells cultured. A sign for such a depletion is the acidification of the culture medium. In this case, phenol red, a constituent of most media formulations, turns yellow.*

4. Evaluate cellular viability after 10 to 14 days using, for example, the Cell Proliferation Kit I (MTT)*, the Cell Proliferation Kit II (XTT)*, or the Cell Proliferation Reagent WST-1*.
   - Alternatively, 200 to 500 cells may be plated in 1 to 2 ml culture medium containing Hygromycin B as described, into Petri dishes (tissue-culture grade, 35 mm), and incubated for 10 to 14 days.
   - The cytotoxic effect may be determined by evaluating the number of surviving cell colonies or percent confluency.

Selection of Transfected Cells

Selection of cells transfected with a DNA construct encoding for hygromycin resistance is performed, using culture medium containing Hygromycin B in a concentration determined for the particular experimental setup as described in section, **Determination of Hygromycin B Concentration**.

1. Prepare culture medium containing Hygromycin B as determined with non-transfected cells as described in section, **Determination of Hygromycin B Concentration**.

2. Remove culture medium and add 5 to 6 ml fresh culture medium (containing Hygromycin B) to a 60 mm culture dish containing the freshly transfected cells.
   - Suspension cells are centrifuged 10 minutes, 250 × *g* using a sterile centrifugation tube prior to removal of medium, and resuspended in approximately 5 ml culture medium containing Hygromycin B.

3. After 5 to 7 days, replace medium with fresh culture medium containing Hygromycin B as described, if required.

4. Incubate cells for an additional 5 to 7 days.

5. After incubation, the cell cultures will contain only living cells expressing the hygromycin B-resistant phenotype.
   - Therefore, culture medium containing Hygromycin B may be replaced with fresh culture medium as described above, but without addition of Hygromycin B.

Subculture of Adherent Cells

Depending on transfection efficiency and proliferation kinetics of the cells being transfected, it may be necessary to subculture adherent cells. This is of particular importance with adherent cells, since cells killed by Hygromycin B do not necessarily detach from the culture substrate.

1. After a subcultivation step, only viable cells will adhere to the culture substrate. This facilitates the evaluation of the cultures and optimizes the culture conditions for the surviving cells.
   When Trypsin is used, subcultivation may be performed without changing the culture dish, by carefully removing the trypsin solution using a fine-tipped Pasteur pipette, carefully leaving the cells behind.

2. Resuspend cells in 6 ml culture medium containing Hygromycin B and serum or a trypsin inhibitor.

3. Incubate cells in the same culture dish.
   - For suspension cells, a dilution of cells may be necessary.

Media Replacement

A replacement of culture media containing Hygromycin B after 5 to 7 days is only necessary if nutritional compounds are supposed to be depleted by the cells cultured. A sign of such depletion is the acidification of the culture medium. In this case, phenol red, a constituent of most media formulations, turns yellow.
To continue the selection process for a longer period, continue cultivation of cells in culture medium containing Hygromycin B, as described.

Maintenance of Hygromycin-Resistant Phenotype

For maintenance of the hygromycin-resistant phenotype of established transfected cell lines and for elimination of revertants, cells may be regularly cultured in culture medium containing Hygromycin B at the same concentration used for the initial selection.
Alternatively, the occurrence of revertants may be avoided by permanently culturing the cells in Hygromycin B containing culture medium. In the latter case, from the dose response curve as determined in section, **Determination of Hygromycin B Concentration**, a subtonic Hygromycin B concentration may be chosen.

Cloning of Transfected Cells

After successful transfection and selection, Hygromycin B-resistant transfectants may be cloned. Single-cell cloning ensures that Hygromycin B-resistant transfectants are derived from the same parental cell. Several methods for single-cell cloning, for example, by limiting-dilution or by picking individual cell colonies, may be employed.
For cloning, single-cell suspensions are to be prepared and cells are plated at low densities. Therefore, adherent cells are to be subcultured. Even though every attempt is made to ensure that the cells are in single-cell suspension prior to plating, it is not guaranteed that colonies do not arise from two cells sticking together.
Therefore, cloning should be done at least twice ("recloning") to generate a clonal population.

Additional Medium Required

The media formulations used are the same as for the standard culture of the respective cell type or for the selection procedure. Whether or not Hygromycin B is added is to be decided according to the particular experimental setup, as described above.

Cloning by Limiting Dilution

The transfectants should be healthy and rapidly proliferating at the time of cloning.

1. Plate cells into a multiwell culture plate (tissue-culture grade, 96- or 24-wells), according to the particular application, such that approximately 1 cell will be plated per well in a final volume of 200 ml culture medium for 96-well plates or 1 ml culture medium for 24-well plates, respectively.

2. Incubate cultures according to their respective requirements.
   - Clones will appear within several days and should be subcultured or passaged according to the particular needs of the cell type cultured, for example, when reaching confluency.

3. Prior to dilution and plating steps, carefully resuspend cell suspensions.

Cloning by Picking

1. Plate adherent cells into Petri dishes (60 mm or 100 mm, tissue-culture grade) at a density of approximately 5 × 10^3 cells for a 60 mm Petri dish or 1 to 1.5 × 10^4 cells for a 100 mm Petri dish, respectively.

2. After several days, colonies of cells (3 to 10 cells) will appear.

3. Incubate cultures according to their respective needs.

4. After several days, colonies of cells (3 to 10 cells) will appear.

5. With the aid of an inverted microscope, the tip of a fire-polished Pasteur pipette is placed adjacent to the selected colony, and cells from the colony are "picked" by suction.

6. Picked cells are transferred into fresh culture dishes and subsequently cultured according to their respective requirements.
   - When cloning by picking is to be performed with suspension cells, plating of cells in soft agar is recommended.

2.3. Parameters

Chemical Formula

$C_{20}H_{37}N_3O_{13}$

Chemical Name

Chemical Structure

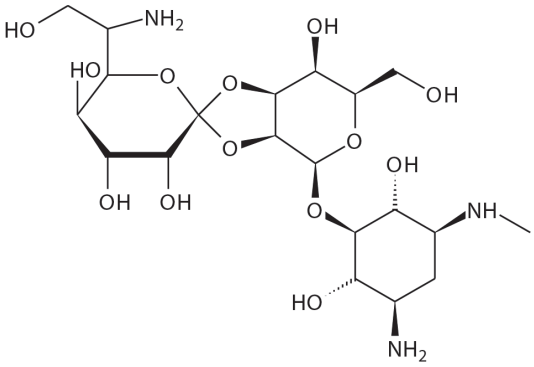

Fig. 1: Chemical structure of Hygromycin B.

Molecular Weight

527.5 Da

Purity

>80% (HPLC)

Working Concentration

Toxic Concentration

Hygromycin B is added to the culture medium at a concentration that varies with the cell type transfected. A titration experiment for each cell type may therefore be performed to determine the amount of Hygromycin B necessary to kill non-transfected cells. A range between 50 µg/ml and 1 mg/ml should be tested.

Working Concentration

The recommended concentration for the selection of resistant cells is 50 to 1,000 µg/ml (see Table). A commonly used concentration for the selection of mammalian cells is 200 µg/ml.

The optimal concentration must be tested experimentally and may vary with the cell type.

The following table shows the concentration of Hygromycin B used for selection of cells after transfection with DNA constructs encoding for hygromycin resistance as taken from the literature.

Species	Cell Type	Cell Line/Strain	Hygromycin B (µg/ml)
E. coli	–	JM 83	25 – 100
			200
Streptomyces lividans	–	–	50
Saccharomyces cerevisiae	–	–	200
Aspergillus nidulans	–	GR1	250
		GR 5	250
		G 191	250
		GB 20	750
		W 1	>1,000
		FGSC 4	>1,000
		FGSC 237	>1,000
Tobacco	–	–	20 – 200
Chicken	–	DT 40	1,500
		27 C 2	1,500
		RP 9	1,500
		HD 3	1,500
		CU 39	1,500
		BM 2	1,500
Mouse	ES cells	LTK	200 – 400
		L 929	200
		C 127	300
		NIH 3T3	50 – 500
		PA 317	50
		ψ 2	150
		Ω E	50
			120
Rat	Embryo fibroblasts	–	200
Mink	–	CCL 64	400
Human	–	B 95-8	50
		Raji	100
		721	100
		WIL 2 TK-/-	200
		Daudi	200
		GG 68	400
		293	200
		K-562	200
Mouse/Human	Hybrid cell	SCC 16-5	200

3. Additional Information on this Product

3.1. Test Principle

How this Product Works

Hygromycin B is an aminoglycosidic antibiotic produced by *Streptomyces hygroscopicus* that kills bacteria, fungi, and higher eukaryotic cells by inhibiting protein synthesis. It has been reported to interfere with translocation and to cause mistranslation.

Resistance

A gene has been identified that confers resistance in *E. coli* against Hygromycin B. The resistance gene codes for kinase (hygromycin B phosphotransferase) that inactivates Hygromycin B through phosphorylation.
Cloning of the resistance gene (designated HM+, HMR, hyg, or hph) and fusion with eukaryotic promoters has resulted in the construction of vectors that allow selection for resistance to Hygromycin B in both prokaryotic and eukaryotic cell systems. A variety of vectors have been developed.

G-418 Solution

G-418 Solution* is another type of aminoglycoside antibiotic commonly used for selection of transfected eukaryotic cells.
This antibiotic can be inactivated by the bacterial aminoglycoside phosphotransferases APH(3′)II and APH(3′)I encoded by genes on transposons Tn5 and Tn601 (also known as Tn903), respectively. Transfection of the neomycin resistance gene(s) (neo) from transposon Tn5 or Tn601 into cells results in resistance to G-418 Solution (neo^r) and enables the cells to grow in media containing G-418 Solution. Resistance to neomycin and to hygromycin can be selected for independently and simultaneously in cell lines that have been transfected with both genes. Thus, two different vectors can be introduced into one cell line, either simultaneously or sequentially.

4. Supplementary Information

4.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

4.2. Changes to previous version

Layout changes.
Editorial changes.
Update to include new safety Information to ensure handling according controlled conditions.

4.3. Ordering Information

Product	Pack Size	Cat. No.
Reagents, kits		
Cell Proliferation Reagent WST-1	8 ml, 800 tests	05 015 944 001
	25 ml, 2,500 tests	11 644 807 001
G-418 Solution	20 ml, 1 g	04 727 878 001
	100 ml, 5 x 20 ml	04 727 894 001
Cell Proliferation Kit II (XTT)	1 kit, 2,500 tests	11 465 015 001
Cell Proliferation Kit I (MTT)	1 kit, 2,500 tests	11 465 007 001

4.4. Trademarks

All product names and trademarks are the property of their respective owners.

4.5. License Disclaimer

For patent license limitations for individual products please refer to:
List of biochemical reagent products.

4.6. Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

4.7. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

4.8. Contact and Support

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support Site**.

To call, write, fax, or email us, visit **sigma-aldrich.com**, and select your home country. Country-specific contact information will be displayed.

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